Acclimation of Microalgae to Wastewater Environments Involves Increased Oxidative Stress Tolerance Activity

Running head: Acclimation of microalgae to wastewater

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Acclimation of Microalgae to Wastewater Environments Involves Increased Oxidative Stress Tolerance Activity

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Abbreviations: APX, ascorbate peroxidase; BOD, biochemical oxygen demand; DF, discriminant function; DFA, discriminant function analysis; FTIR, Fourier transform infrared; JM, Jaworski's medium; N, nitrogen; OD, optical density; P, phosphorus; PC, principal component; PCA, principal component analysis; PC-DFA, principal component-discriminant function analysis; ROS, reactive oxygen species; TAP, tris-acetate-phosphate.

Abstract

A wastewater environment can be particularly toxic to eukaryotic microalgae. Microalgae can adapt to these conditions but the specific mechanisms that allow strains to tolerate wastewater environments are unclear. Furthermore, it is unknown whether the ability to acclimate microalgae to tolerate wastewater is an innate or species-specific characteristic. Six different species of microalgae (*Chlamydomonas debaryana*, *Chlorella luteoviridis*, *Chlorella vulgaris*, *Desmodesmus intermedius*, *Hindakia tetrachotoma*, *Parachlorella kessleri*) that had never previously been exposed to wastewater conditions were acclimated over an eight week period in secondary-treated municipal wastewater. With the exception of *C. debaryana*, acclimation to wastewater resulted in significantly higher growth rate and biomass productivity. With the exception of *C. vulgaris*, total chlorophyll content was significantly increased in all acclimated strains, while all acclimated strains showed significantly increased photosynthetic activity. The ability of strains to acclimate was species-specific, with two species, *C. luteoviridis* and *P. kessleri*, able to acclimate more efficiently to the stress than *C. debaryana* and *D. intermedius*. Metabolic fingerprinting of the acclimated and non-acclimated microalgae using Fourier transform infrared spectroscopy was able to differentiate strains on the basis of metabolic responses to the stress. In particular, strains exhibiting greater stress response and altered accumulation of lipids and carbohydrates could be distinguished. The acclimation to wastewater tolerance was correlated with higher accumulation of carotenoid pigments and increased ascorbate peroxidase activity.

Keywords: acclimation; ascorbate peroxidase; Fourier transform infrared spectroscopy; microalgae; oxidative stress; wastewater

Introduction

Microorganisms have colonised and exploited ecological niches across the planet, with many of these being extreme environments. Survival in such variable environments requires the ability to rapidly adapt and tolerate to stress. Understanding the mechanisms of how microorganisms respond and adapt to environmental stresses is important in order to explain the patterns of microbial diversity seen across the planet and to understand the plasticity of many organisms to variable environments (Nevo 2011). While many of the adaptive mechanisms of bacterial and fungal microbes are quite well understood, much less is known regarding the adaptation processes of eukaryotic photosynthetic microorganisms. Some studies have begun to indicate whether short-term adaptation of microalgae to a stress exposure is due to genetic changes caused by spontaneous mutation, and thus often heritable, or by physiological adaptation (referred to as acclimation), which may not be replicated in the progeny. For example, adaptation of microalgae to severe doses of antibiotics, herbicides, and mine waste (Sánchez-Fortún et al. 2009; Huertas et al. 2010; García-Balboa et al. 2013) were found to be due to genetic adaptation, while exposure to a lower dose of herbicide did not have a genetic basis (Marvá et al. 2010). Indeed, short-term adaptation of microalgae due to rare mutations is usually only observed following exposure to acute stress. In contrast, microalgae have been shown to acclimate to a variety of sub-lethal stresses, like heavy metals, singlet oxygen, salinity and high light (Johnson et al. 2007; Ledford et al. 2007; Bonente et al. 2012; Perrineau et al. 2014). However, in many cases the specific physiological mechanisms underlying the acclimation process are unknown.

Wastewater is an environment that can be particularly toxic to microalgae and many strains, including culture collection strains not previously exposed to such environments, have been observed to show sensitivity to wastewaters from various sources (Kong et al. 2010; Li et al. 2011). Wastewater toxicity can be due to multiple factors and depends on the source of waste and the type of wastewater (Pittman et al. 2011). For example, with municipal sewage wastewater, factors including bacterial burden, high ammonium concentration, heavy metals and high oxygen concentration can cause significant toxicity (Ip et al. 1982; Konig et al. 1987; Pearson et al. 1987; Wrigley and Toerien 1990), and can lead to the formation of reactive oxygen species (ROS) within the microalgal cell (Osundeko et al. 2013). Despite the toxicity of wastewater to microalgae, there are strong applied interests in the successful cultivation of microalgae in wastewater; for example, as a novel method for pollutant

bioremediation (Craggs et al. 2011). Wastewater could also be used as a nutrient-rich cultivation medium for sustainable microalgal biomass production, such as for biofuel use, thus mitigating the need to input scarce resources like freshwater and fertiliser (Pittman et al. 2011; Driver et al. 2014). Furthermore, the dual potential of wastewater-grown microalgae for biofuel and remediation offers economic advantage to the strategy of cultivating microalgae in wastewater (Lundquist et al. 2010). The identification of wastewater-tolerant strains is therefore of interest. Microalgae strains have been identified that can tolerate and grow in such environments (McGinn et al. 2012; Wu et al. 2012; Osundeko et al. 2013). For example, two strains, *Chlorella luteoviridis* and *Parachlorella hussii*, were identified as indigenous, naturally-adapted strains to secondary-treated wastewater (Osundeko et al. 2013) that can tolerate more toxic wastewater, including the centrate liquor from the dewatering of the activated sludge (Osundeko and Pittman 2014). In addition, strains acclimated to wastewater have the ability to provide enhanced nitrogen (N) and phosphorus (P) removal efficiencies (Lau et al. 1996).

However, the specific mechanisms that allow selected microalgae to acclimate to wastewater environments are not known. Furthermore, it is unknown whether the ability to acclimate chlorophyte microalgae to tolerate growth in wastewater is an innate characteristic or a species-specific characteristic. Here we have taken six strains of microalgae from a culture collection (*Chlamydomonas debaryana*, *C. luteoviridis*, *Chlorella vulgaris*, *Desmodesmus intermedius*, *Hindakia tetrachotoma* and *Parachlorella kessleri*) that were never previously exposed to wastewater but are either identical or closely related to species previously identified in wastewater environments (Jimenez-Perez et al. 2004; Zhou et al. 2011; Wu et al. 2012; Osundeko et al. 2013). We then assessed their ability to acclimate to wastewater, identified species-specific differences, and evaluated the potential mechanisms of tolerance behind these differences using a range of physiological, biochemical and metabolic responses.

Results

Acclimation of selected microalgae strains to wastewater

Six strains of chlorophyte microalgae obtained from a culture collection with no history of growth in a wastewater environment, showed low cell density when exposed to raw municipal secondary-treated

wastewater, with particularly poor growth for *C. debaryana*, *C. vulgaris*, *D. intermedius* and *H. tetrachotoma* (Fig. 1A). Furthermore, growth rate in wastewater was substantially reduced compared to growth in an artificial medium (Fig. 1B). To investigate whether the growth of the strains in wastewater could be improved by short-term acclimation, the strains were exposed to the effluent over an 8 week period of successive 7 d batch culture phases. Quantification of cell density at the end of each week showed increases in optical density (OD), with particularly marked rises in cell density for *C. luteoviridis* and *P. kessleri* (Fig. 1A). With the exception of *C. debaryana*, growth rate of the acclimated strains in untreated wastewater was significantly higher than the non-acclimated strains, with acclimated *C. luteoviridis* and *P. kessleri* yielding growth rate values equivalent to those of the same strains grown in artificial medium (Fig. 1B). This greater tolerance to the wastewater by some of the acclimated strains, notably *C. luteoviridis* and *P. kessleri*, and to a lesser extent *C. vulgaris* and *H. tetrachotoma* was also clear from visual inspection of the culture flasks (Supplementary Fig. S1). Biomass productivity also increased in most of the acclimated strains, again highly in the *C. luteoviridis* and *P. kessleri* cultures (Fig. 1C). The increase in growth rate and biomass productivity was also observed if the wastewater was autoclaved, for all of the strains except for *C. debaryana* and *D. intermedius* (for biomass productivity) (Supplementary Fig. S2), suggesting that the tolerance gained by the acclimation is not just related to microbial contamination of the wastewater.

Changes in concentrations of photosynthetic pigments, and measurement of chlorophyll fluorescence, were used as indicators of environmental stress. Total chlorophyll content of the acclimated and non-acclimated strains in raw wastewater was significantly higher in all of the acclimated strains except for *C. vulgaris*, although there was variation in total chlorophyll between strains, with *C. debaryana* and *D. intermedius* having the lowest value, particularly in the non-acclimated strains (Fig. 2A). Carotenoid concentration was significantly higher in the acclimated *P. kessleri* strain compared to the non-acclimated strain (Fig. 2B). However, differences observed in the other strains were found not to be significant. Carotenoid concentration was very low in both sets of *C. debaryana* and *D. intermedius* strains. The F_v/F_m ratio, which is a relative measure of maximal Photosystem II (PSII) quantum yield and a determinant of PSII activity, was very low in the non-acclimated *C. debaryana* and *D. intermedius* and markedly increased after these strains had been acclimated (Fig. 2C). Acclimation increased F_v/F_m ratio in all the other strains but the change was substantially less, particularly for *C. luteoviridis* and *P. kessleri*.

Metabolic fingerprinting of acclimated versus non-acclimated strains

Metabolic profiles of the acclimated and non-acclimated strains were used to identify metabolic responses of the strains. Fourier transform infrared (FTIR) spectroscopy coupled to multivariate statistical clustering was used as a rapid and powerful method to generate metabolic fingerprints (Ellis et al. 2002; Allwood et al. 2006; Dean et al. 2010). FTIR spectra were obtained from acclimated and non-acclimated strains grown in artificial medium then exposed to raw wastewater after 4 d (early exponential phase growth) and after 10 d (late exponential/early stationary phase growth). Visual comparisons of spectra over the wavenumber range 1760 – 950 cm^{-1} showed high similarity between replicate spectra while spectra of acclimated and non-acclimated strains were also very similar. However, there were some clear differences for some of the species, particularly for *C. debaryana* at day-4 and day-10, and *D. intermedius* at day-10 (Supplementary Fig. S3). Chemometric analysis was used to assess statistically whether there was metabolic differentiation between the species, and between acclimated and non-acclimated strains. Furthermore, this analysis was used to determine the metabolites that explain any differentiation, as specific bands in the FTIR spectra can be associated with known macromolecules and metabolites on the basis of biochemical standards and previous published studies (Supplementary Fig. S4).

An unsupervised principal component analysis (PCA) was not able to show clear separation between the FTIR spectra obtained at day-4 and day-10, apart from individual clustering of non-acclimated *C. debaryana* at day-4 (Supplementary Fig. S5A), and clustering of non-acclimated *D. intermedius* and two of the non-acclimated *C. debaryana* replicates at day-10 (Supplementary Fig. S5C). The principal component (PC) loadings for the day-4 spectra indicates that PC2, which explains the variation between non-acclimated *C. debaryana* and the other samples, was due to less carbohydrate in these strains (particularly at 1086, 1050 and 1036 cm^{-1}) and increased lipid (at 1745 cm^{-1}), plus an increase in a protein peak at ~1300 cm^{-1} (Supplementary Fig. S5B). Furthermore, PC loadings for the day-10 spectra show that most of the variation was due to PC1, explained predominantly by changes in carbohydrate (Supplementary Fig. S5D). Because of the generally weak differentiation of spectra by PCA, a supervised discriminant function analysis (DFA) was performed on the PCs, with the PC-DFA plot from the day-4 spectra showing clear clustering in two distinct clusters

of non-acclimated *C. debaryana* and non-acclimated *D. intermedius* from the rest of the samples, which were all tightly grouped (Fig. 3A). The PC-DFA loadings again indicate that the variation between non-acclimated *C. debaryana*, non-acclimated *D. intermedius*, and the other samples was due mainly to lipid, protein and carbohydrate changes (Fig. 3B). The PC-DFA plot of the day-10 spectra also showed some distinct clusters, with non-acclimated *C. debaryana* and *D. intermedius* again separated from the acclimated samples, but also non-acclimated *H. tetrachotoma* separated from the acclimated samples (Fig. 3C). There was also some separation between acclimated and non-acclimated *C. luteoviridis* and *C. vulgaris* but both sets of *P. kessleri* samples were tightly clustered. Most of the variation in this plot (DF1) was due predominantly to changes in carbohydrate, principally the 1050 and 1036 cm^{-1} wavenumber bands (Fig. 3D). Overall, this data shows that distinct metabolic fingerprints can be determined by FTIR of acclimated versus non-acclimated strains for some but not all species, with differences in carbohydrates and lipids explaining the majority of the variation.

Relative total carbohydrate and lipid concentrations were therefore quantified from the ratio of the mean absorbance values of the carbohydrate (1200 - 900 cm^{-1}) bands with the amide I (1655 cm^{-1}) band, and the lipid (1745 cm^{-1}) band with the amide I band at day-4 (Supplementary Fig. S6) and day-10 (Fig. 3E, F). At day-4 differences in carbohydrate and lipid abundance between the acclimated and non-acclimated strains were relatively minor. There were only significant changes in the carbohydrate/amide ratio for *C. debaryana*, which was markedly increased following acclimation, and for *H. tetrachotoma* and *P. kessleri*, where values were slightly reduced following acclimation (Supplementary Fig. S6A). Furthermore, there were significant but minor reductions in lipid/amide ratio values for *C. luteoviridis*, *H. tetrachotoma* and *P. kessleri* following acclimation, and a slight increase for acclimated *D. intermedius* (Supplementary Fig. S6B). At day-10, changes in carbohydrate and lipid were clearly apparent for *C. debaryana* and *D. intermedius*, with substantial reduction in carbohydrate/amide ratio for both acclimated strains (Fig. 3E), and a large reduction in lipid/amide ratio for acclimated *C. debaryana* (Fig. 3F). Comparisons of acclimated and non-acclimated strains of the other species at day-10 were either not significantly different or showed only minor changes. Although lipid/amide ratio values showed little change between acclimated strains, lipid productivity (g L^{-1} d^{-1}) values determined at day-10 were significantly higher in three of the acclimated strains, *D. intermedius, C. luteoviridis* and *P. kessleri* (Fig. 6A), with the latter two strains showing a particularly high increase, indicating that lipid productivity was largely determined by biomass production.

Ability of acclimated and non-acclimated strains to utilise acetate

Acetate utilisation in the acclimated and non-acclimated strains was examined by quantifying the growth of strains grown in artificial media either with or without 0.1% acetate. All strains, both acclimated and non-acclimated, grew significantly better as determined by cell density measurement in the presence of acetate compared to without acetate (Fig. 4). Although the cell density in the presence of acetate was higher for the acclimated strains of all species compared to the non-acclimated strains, the relative increase in cell density (acetate growth versus no acetate growth) was not significantly different between both sets of strains, with 3- to 4-fold increases in OD values. The only exception was *C. vulgaris*, which did show a significantly higher fold-increase in cell density in the presence of acetate for the acclimated strain (5.4-fold) compared to the non-acclimated strain (3.5-fold).

Increased ascorbate peroxidase activity in acclimated strains

To examine whether the ability to tolerate and mitigate ROS accumulation was altered in the acclimated strains, ascorbate peroxidase (APX) activity, which is an important anti-oxidant enzyme in many microalgae species (Tanaka et al. 2011; Urzica et al. 2012), was quantified in strains either transferred from artificial medium into fresh artificial medium (non-stressed control conditions) or transferred from artificial medium into wastewater. Background APX activity in all strains kept under non-stressed conditions in artificial medium was very low but exposure to wastewater mediated a significant increase in APX activity in all strains (Fig. 5). However, induction of the APX activity was greatly enhanced in all of the acclimated strains compared to the non-acclimated strains, with highest activity in the acclimated *P. kessleri* strain, slightly reduced activity in the acclimated *C. luteoviridis*, *C. vulgaris* and *H. tetrachotoma* strains, but low APX activity in the acclimated *C. debaryana* and *D. intermedius* strains. For these latter two strains, APX activity was significantly higher compared to that seen in the non-acclimated strains, but the increase was not substantial. It was also apparent that wastewater-induced APX activity in the non-acclimated *P. kessleri* strain was significantly higher than in all of the other non-acclimated strains (Fig. 5).

Impact of acclimation on wastewater pollutant remediation capability

The pollutant remediation capability of the acclimated and non-acclimated strains in wastewater were tested by measuring the biochemical oxygen demand (BOD) reduction, and NH_4^+-N and PO_4^{3-}-P removal. All acclimated and non-acclimated strains were tested except *C. vulgaris*. Initial PO_4^{3-}-P, NH_4^+-N and BOD values of the wastewater were 1.03 ± 0.14 mg L^{-1}, 25.06 ± 3.1 mg L^{-1} and 13.42 ± 0.59 mg L^{-1}, respectively. BOD reduction was significantly enhanced (>70%) in all acclimated strain cultures, compared to low reduction (<40%) by the non-acclimated strains, except for *C. debaryana*, which was not as efficient although BOD reduction by non-acclimated *C. debaryana* was higher than for the other non-acclimated strains (Fig. 6B). The PO_4^{3-}-P and NH_4^+-N removal rates were significantly higher in all of the acclimated strain cultures than the non-acclimated cultures, with more than 75% of PO_4^{3-}-P and 60% of NH_4^+-N removed by the acclimated strains (Fig. 6C, D). Although, acclimation did not significantly improve *C. debaryana* and *D. intermedius* growth in untreated wastewater (Fig. 1), their capability to remove PO_4^{3-}-P and NH_4^+-N were significantly improved. However, it worth noting that the background microbial contribution to the removal of N and P and reduction of BOD was not quantified in this study.

Discussion

Although wastewaters contain biotic and abiotic constituents that can negatively impact microalgae growth, indigenous municipal wastewater strains, including *C. luteoviridis*, *H. tetrachotoma* and *P. hussii*, have previously been identified to grow well in this environment and their strong growth was attributed partly to tolerance to oxidative stress (Osundeko et al. 2013). However, it was unclear whether this wastewater tolerance was due to characteristics specific to these species or whether this tolerance was due to a general, non-species specific adaptation (or acclimation) to the wastewater environment. By comparing the growth and physiology of six different culture collection microalgae species in wastewater before and after a short acclimation period, it is possible to conclude that acclimation can substantially improve tolerance to this stressful environment; however, there is a clear indication that species-specific characteristics are also a key factor.

While all species displayed improved growth after acclimation (Fig. 1A), the improvement of two species, *C. debaryana* and *D. intermedius*, was clearly reduced. The acclimated strains of these species either displayed the lowest or no significant increase in growth rate, biomass productivity, and chlorophyll content. Interestingly, the increase in F_v/F_m ratio was the most substantial for these two strains, indicating that they had undergone physiological acclimation even though growth parameters were not significantly improved. In contrast, two of the other species, *C. luteoviridis* and *P. kessleri*, showed substantial tolerance to the wastewater following acclimation; however, it was also clear that even the non-acclimated strains of these species had higher tolerance to the wastewater, and in particular, non-acclimated *P. kessleri* was as tolerant as many of the other acclimated strains. Previously, we found that a related species *P. hussii* isolated from a secondary-treated effluent tank, had very high stress tolerance characteristics (Osundeko et al. 2013). Other studies have found strains of this genus to be tolerant to extreme conditions. For example, *P. kessleri* isolated from wastewater effluent was reported to have high propensity for growth in saline, high temperature, oxidative, acidic and alkaline conditions and efficient in accumulating radionuclide particles (Shimura et al. 2012). Likewise, a strain of *P. kessleri* was identified that can tolerate an extremely acidic geothermal pond (Juárez et al. 2011).

Metabolic profiling of the acclimated and non-acclimated microalgae using FTIR spectroscopy clearly demonstrated that specific strains could be differentiated on the basis of metabolic responses to the wastewater stress. In particular, strains exhibiting greater stress response and altered accumulation of lipids and carbohydrates could be distinguished. Thus there was a strong separation by PC-DFA of non-acclimated *C. debaryana* and *D. intermedius* from the other strains. As these were the strains that also showed weak physiological parameters such as low chlorophyll content and low F_v/F_m ratio (Fig. 2A, C), we can conclude that the metabolic changes within these cells, notably carbohydrate and lipid accumulation (Fig. 3E, F), are indicative of a stress response. Many environmental stresses, such as nutrient limitation or salinity, have been shown to induce the accumulation of storage carbon metabolites, principally triacylglycerol and starch, in microalgae, which are maintained as energy stores during periods of restricted growth (Hu 2007; Dean et al. 2010; Perrineau et al. 2014). In addition, many microalgae accumulate phospholipids for signalling roles and osmoregulatory metabolites such as glycerol in response to stresses such as salinity (Ginzburg et al. 1995; Arisz and Munnik 2011). Thus it appears that for some microalgae species, wastewater stress is

also able to elicit such general metabolic stress responses. It was also apparent from the metabolic profiling that strains, such as *P. kessleri*, which were efficiently acclimated and showed strong tolerance to the wastewater could not be distinguished on the basis of acclimation, particularly during the early stage of growth (Fig. 3A). This therefore demonstrates that the acclimation process did not induce significant metabolic remodelling of the cells and suggests that specific metabolites do not play a major role in the mechanism of wastewater acclimation. This is in contrast to the mechanisms of acclimation to stresses such as high light, where modification of primary and secondary metabolism appears to be important for photoacclimation in *Chlamydomonas reinhardtii* (Davis et al. 2013).

Various other characteristics were therefore examined in order to attempt to understand the mechanistic basis of the acclimation process. Increased efficiency in the utilisation of organic carbon, which is rich in wastewater, was not found to be a general explanation, as there was no difference in growth on acetate between acclimated and non-acclimated strains, except to an extent for *C. vulgaris*. However, it is important to note that there is a large variation in the types of dissolved organic matter and dissolved organic carbon present in wastewater effluent that could be tested (Imai et al. 2002). Bacterial tolerance does not appear to be an important factor as the non-acclimated strains were as sensitive to autoclaved wastewater as to the raw untreated wastewater. In contrast, two characteristics that correlated strongly with the improved tolerance in the acclimated strains were an increase in APX activity and an accumulation of carotenoid pigments. As carotenoid content was low in the wastewater-stressed strains *C. debaryana* and *D. intermedius*, and elevated in wastewater-tolerant acclimated strains such as *C. luteoviridis* and *P. kessleri*, rather than being an indicator of stress, we suggest that the carotenoids are involved in the tolerance mechanism. Carotenoids such as beta-carotene and astaxanthin have been shown to provide protective functions against oxidative stress induced by adverse environmental conditions including high light and salinity (Ben-Amotz et al. 1989; Li et al. 2008; Lemoine and Schoefs 2010). Therefore carotenoid accumulation in wastewater-grown microalgae may provide tolerance against ROS that accumulate under wastewater conditions (Osundeko et al. 2013). This is analogous to the accumulation of carotenoids including zeaxanthin, a potent ROS scavenger, observed in *C. reinhardtii* acclimated to high light conditions (Bonente et al. 2012). In a similar manner, increased APX activity will be expected to allow the microalgae to tolerate oxidative stress more efficiently. Although APX activity was enhanced in all strains exposed to wastewater, activity was increased in all acclimated strains, but most significantly in *P. kessleri* (Fig.

5). High APX activity was previously shown in indigenous wastewater-tolerant strains of *C. luteoviridis* and *P. hussii*, at levels equivalent to seen here in the acclimated *C. luteoviridis* and *P. kessleri* (Osundeko et al. 2013). Rapid induction of anti-oxidant enzymes may be a characteristic of short-term acclimation to oxidative stress conditions. For example, short-term acclimation to salt stress or singlet oxygen stress in *C. reinhardtii* has been found to induce the up-regulation of genes involved in ROS scavenging (Ledford et al. 2007; Perrineau et al. 2014).

This study provides evidence that an acclimation process is a potentially useful approach for enhancing algae tolerance to wastewater cultivation for bioremediation purposes and/or biofuel production. For example, the acclimated strains were shown to have significantly improved N, P and BOD removal efficiency and increased lipid productivity than non-acclimated strains (Fig. 6). While naturally adapted, indigenous strains that already tolerate wastewater conditions could be used for such purposes, not all strains may be suitable for industrial applications; for example, if they have slow growth, poor pollutant accumulation, or poor biofuel feedstock characteristics. Modifying microalgal strains by genetic engineering would not be suitable for commercial use due to regulatory restrictions and environmental concerns (Flynn et al. 2013). Therefore the use of an acclimation process could serve as a way to improve the wastewater tolerance of any non-indigenous strain that has other useful characteristics. This study therefore opens up a new method of improving the spectrum of microalgae capability in wastewater cultivation.

Materials and Methods

Microalgae strains and culture conditions

Microalgae strains were obtained from the UK Culture Collection of Algae and Protozoa (CCAP): *Chlamydomonas debaryana* (CCAP 11/70; originated from spruce forest soil in the former Czechoslovakia), *Chlorella luteoviridis* (CCAP 211/3; originated from freshwater in the Netherlands), *Chlorella vulgaris* (CCAP 211/79; origin unknown), *Desmodesmus intermedius* (CCAP 258/38; originated from a freshwater lake in Germany), *Hindakia tetrachotoma* (CCAP 222/81; originated from a freshwater lake in Kenya) and *Parachlorella kessleri* (CCAP 211/11G; originated from freshwater in USA). All strains were maintained and grown to late exponential phase in Jaworski's Medium (JM)

(Andersen 2005), which has similar concentrations of trace nutrients to the secondary-treated wastewater medium. The original culture collection strains (non-acclimated strains) were maintained in JM. With the exception of the acetate experiments (see below), all cultivation was carried out in municipal secondary-treated wastewater. This wastewater is the out-flow from an activated sludge treatment, and was collected from United Utilities Water plc wastewater treatment plant at Ellesmere Port, Cheshire, UK (lat. 53° 16′ 47.32″ N, long. 2° 53′ 50.65″ W), which receives mainly domestic wastewater and pre-treated effluent from a nearby oil refinery. The wastewater that was used for all cultivation experiments showed natural variability in chemical composition, and contained mean values of 27.2 ± 0.66 mg L^{-1} NH_4^+-N, 17.3 ± 2.05 mg L^{-1} NO_3^--N, 0.94 mg L^{-1} PO_4^{3-}-P, 17.76 ± 2.21 mg L^{-1} suspended solids, 12.78 ± 2.11 mg L^{-1} BOD, 104.03 ± 5.18 mg L^{-1} chemical oxygen demand, and had a mean pH of 7.56. For the remediation analysis, initial values of PO_4^{3-}-P, NH_4^+-N, and BOD were 1.03 ± 0.14 mg L^{-1}, 25.06 ± 3.1 mg L^{-1} and 13.42 ± 0.59 mg L^{-1}, respectively. Prior to using the wastewater, it was allowed to settle and large particles were removed by decanting. All artificial media was autoclaved, while wastewater cultivation was performed using untreated wastewater except in the experiment which evaluated growth in autoclaved wastewater, autoclaved at 121°C for 20 min. Batch cultivation was performed in 200 ml glass flasks in triplicate on an orbital shaker at 2 Hz at 22°C, 16 h light : 8 h dark and a photon flux of 150 µmol m^{-2} s^{-1}. For growth comparisons without or with acetate as a carbon source, strains were grown in TAP (Tris-acetate-phosphate) medium (Harris 1989) containing 0.1 % (v/v) glacial acetic acid or in medium without acetate (TP medium). The starting cell density of each inoculate as determined by optical density at 680 nm (OD_{680}) was 0.05 in each flask.

Acclimation in wastewater

For the acclimation process, microalgae were first cultured in fresh medium containing 50% (v/v) JM and 50% (v/v) autoclaved wastewater for 5 d. A 1ml aliquot from these cultures was then diluted with 10 ml of fresh autoclaved wastewater. A 20 µL sample of this culture was grown on autoclaved wastewater agar plates containing 1.5% (w/v) agar for 14 d. Distinct microalgae colonies from each plate were selected and sub-cultured in untreated wastewater for 7 d to select the most tolerant cells for each strain. Microalgae were then subjected to further acclimation for 8 weeks by continuous subculture in fresh samples of untreated wastewater, at an inoculate volume of 4% every 7 d. At the

start of each weekly cultivation period cells taken from the previous period were inoculated with a starting OD_{680} value of 0.05. OD_{680} was measured for each culture as an indicator for growth at day-7 during each period the 8 week acclimation process. The presence of algae in the culture was routinely monitored by microscopic examination.

Microalgae growth, physiological and biochemical analysis

Cell density of each culture was routinely determined at every 2 d of the cultivation by OD_{680} measurement using a visible light-UV spectrophotometer (Jenway). This is a suitable tool for monitoring algae growth in wastewater and showed a positive correlation with cell number and dry weight biomass (Osundeko et al. 2013). Prior to OD measurement, wastewater without algae was used as a blank. Specific growth rates, determined between day-2 and day-8, dry weight biomass, and biomass productivity, were determined as described previously (Osundeko et al. 2013). Total lipid measurement and lipid productivity were determined as described previously (Osundeko et al. 2013; Osundeko and Pittman 2014). For the remediation experiment, NH_4^+-N and PO_4^{3-}-P in the culture media were analysed on a Skalar Sans Plus autoanalyser using standard methodology (Skalar Analytical). BOD measurement was determined using a Jenway 9500 Dissolved Oxygen Meter (Jenway) following incubation at 20 °C for 5 d. All other wastewater data were provided by United Utilities. APX activity was quantified as described previously (Osundeko et al. 2013), except that the strains were grown in TAP medium until late exponential phase then incubated for 24 h in untreated wastewater or TAP medium alone (non-stressed control) before cell harvesting and analysis. A spectrophotometric method (Lichtenthaler 1987) was used to determine relative total chlorophyll and carotenoid content, as used elsewhere for green microalgae (Chang et al. 2013; Melegari et al. 2013). As the absorption maxima of chlorophyll *b* and carotenoids overlap at 470 nm, this method provides a semi-quantitative, relative measurement between acclimated and non-acclimated strains. At the late exponential growth phase ten ml of each of the culture was centrifuged at 1500 *g* for 20 min, and the pellet subjected to a freeze-thaw procedure using liquid N_2 thus ensuring complete extraction of pigment. The pellet was then re-suspended in 5 ml of 80% acetone, incubated for 15 min then re-centrifuged. The extract OD was measured at 470 nm, 663 nm, 646 nm and 750 nm. Total chlorophyll (as determined by addition of chlorophyll *a* and *b*) and total carotenoid content was calculated by the

formula described previously (Lichtenthaler 1987). The chlorophyll fluorescence emission was measured using a Heinz Walz PAM-101 chlorophyll fluorimeter and the ratio F_v/F_m (maximum quantum yield of photosynthesis) was calculated as described (Maxwell and Johnson 2000).

FTIR spectroscopy

A 0.5 ml sample was taken from each replicate flask for each acclimated and non-acclimated strain grown in wastewater at day 4 and day 10, centrifuged at 1500 *g* for 20 min, the supernatant removed and the cells re-suspended in approximately 100 µm of distilled water. A 30 µl sample was then deposited on a 96-well silicon microplate, and oven dried at 40ºC for 1 h. The plate was placed in a HTS-XT high-throughput microplate extension and FTIR spectra collected using an Equinox 55 FTIR spectrometer (Bruker Corporation), equipped with a mercury-cadmium-telluride detector cooled with liquid N_2. Spectra were collected over the wavenumber range 4000-600 cm^{-1}. Each sample was analysed in triplicate. Spectra were pre-processed using extended multiplicative signal correction (Martens and Stark 1991) prior to multivariate analysis. Band assignments were determined as described previously (Giordano et al. 2001; Dean and Sigee 2006; Murdock and Wetzel 2009).

Statistical analysis

Unless otherwise stated, all data shown is representative or mean data of at least three replicate experiments. Differences between strains and/or treatments were assessed using one-way ANOVA performed using IBM Statistics SPSS 20. When significant differences were detected at a confidence level of 95%, the multi-range Tukey's post-hoc test was applied. Multivariate statistical analysis of FTIR spectra was performed using MATLAB. The spectral data was first analysed by PCA then by DFA. DFA then discriminated between groups on the basis of the retained PCs, essentially as described previously (Allwood et al. 2006).

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Figures:

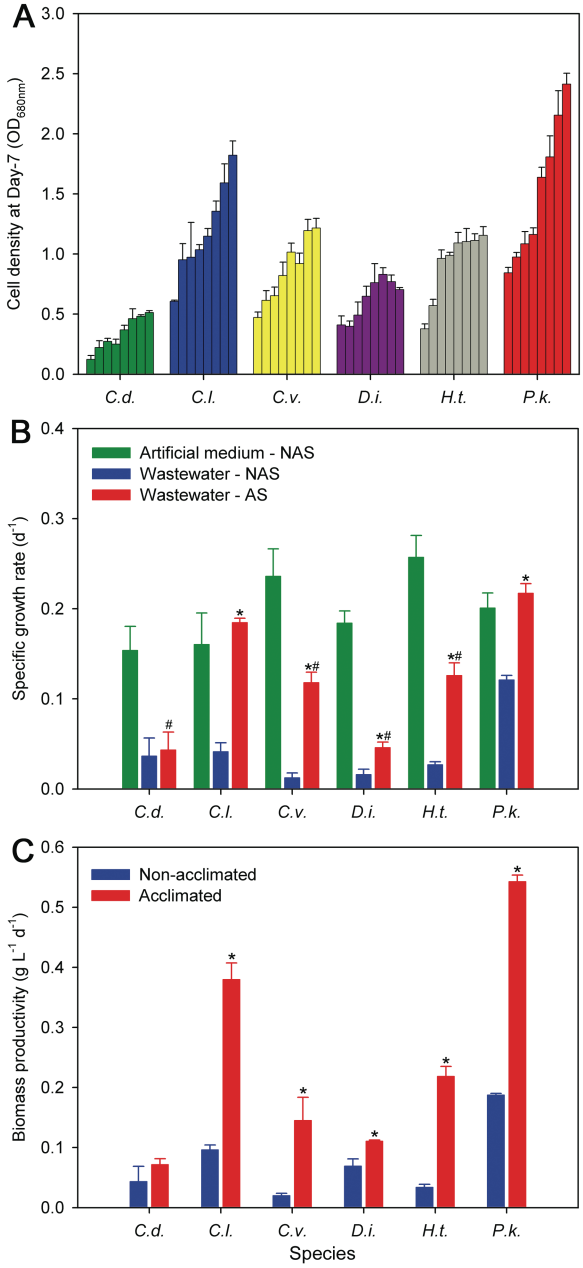

Fig. 1 Acclimation of five microalgae strains to secondary treated wastewater. (A) Cell densities of *C. debaryana* (*C.d.*), *C. luteoviridis* (*C.l.*), *C. vulgaris* (*C.v.*), *D. intermedius* (*D.i.*), *H. tetrachotoma* (*H.t.*) and *P. kessleri* (*P.k.*) taken at day-7 during 8 successive 7 d cultivation periods in wastewater. At the start of each cultivation period cells taken from the previous period were inoculated with a starting OD_{680nm} value of 0.05. (B) Growth rate determined during exponential growth phase of the non-acclimated strains (NAS) in artificial growth medium (JM) and wastewater, and the acclimated strains (AS) in wastewater. (C) Biomass productivity determined during exponential growth phase of the non-acclimated and acclimated strains in wastewater. All data are means ±SE of 3-6 replicates. * (*P* <

0.05) denotes significant difference between AS and NAS in wastewater. # (P < 0.05) denotes significant difference between AS in wastewater and NAS in artificial medium.

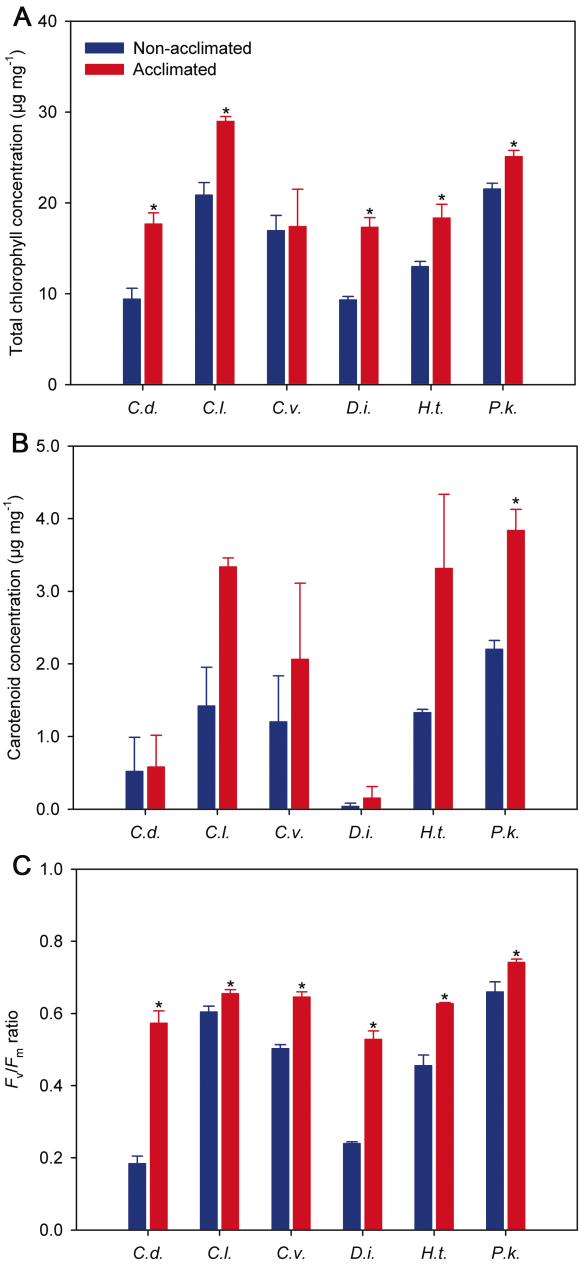

Fig. 2 Photosynthetic pigment analysis and chlorophyll fluorescence of acclimated and non-acclimated strains grown in wastewater. (A-C) Total chlorophyll concentration (Chl *a* + Chl *b*) (A), total carotenoid concentration (B) and maximal PSII quantum yield (F_v/F_m ratio) (C) from non-acclimated and acclimated *C. debaryana* (*C.d.*), *C. luteoviridis* (*C.l.*), *C. vulgaris* (*C.v.*), *D. intermedius* (*D.i.*), *H. tetrachotoma* (*H.t.*) and *P. kessleri* (*P.k.*) determined at day-8 of growth in wastewater. All data are means ±SE of 3 replicates. * (P < 0.05) denotes significant difference between acclimated and non-acclimated strains in wastewater.

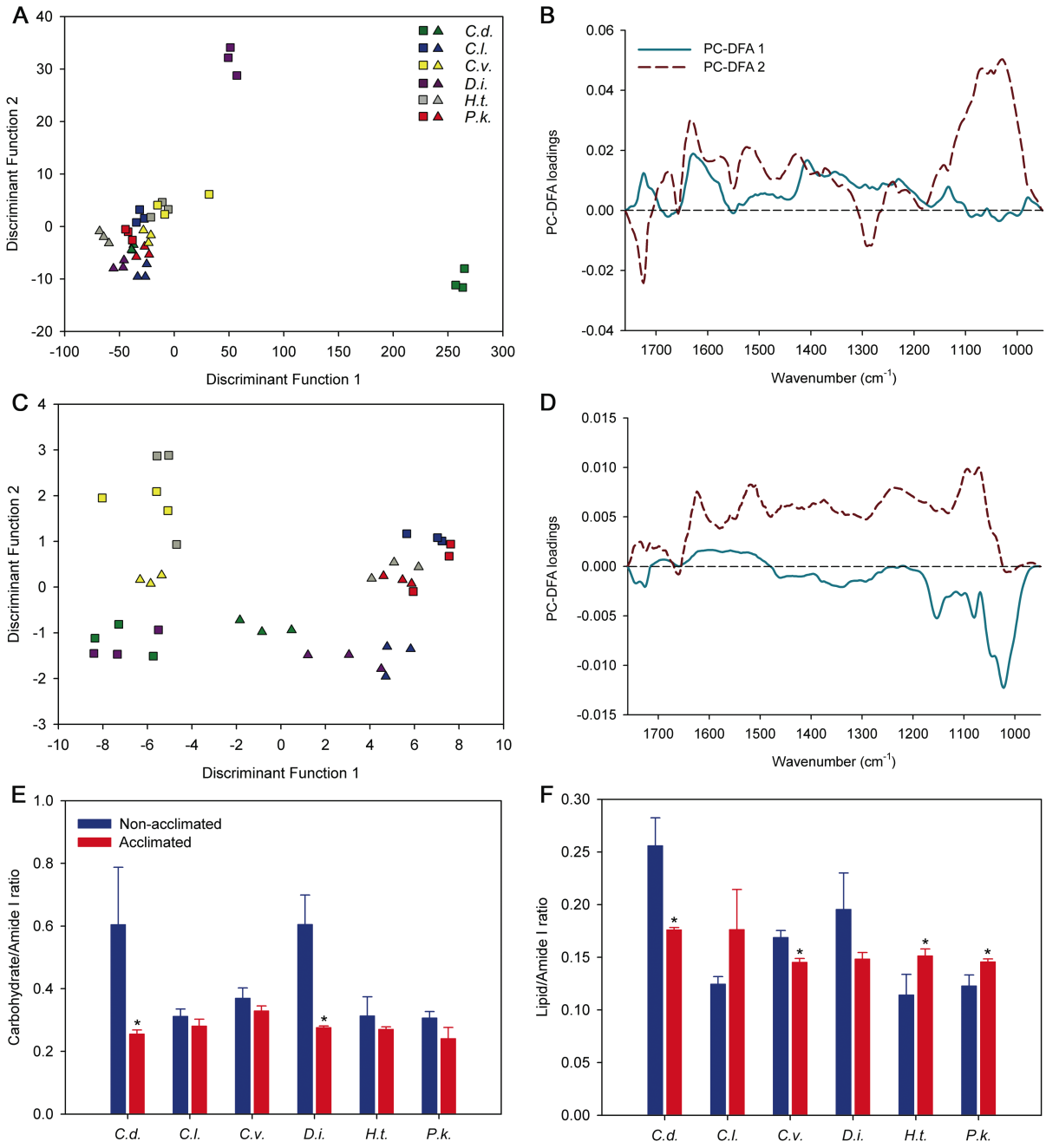

Fig. 3 FTIR spectroscopy analysis of the non-acclimated and acclimated *C. debaryana* (*C.d.*), *C. luteoviridis* (*C.l.*), *C. vulgaris* (*C.v.*), *D. intermedius* (*D.i.*), *H. tetrachotoma* (*H.t.*) and *P. kessleri* (*P.k.*) strains grown in wastewater. (A - D) PC-DFA scores (A, C) and PC-DFA loading plots (B, D) of FTIR spectra of the non-acclimated and acclimated strains at day-4 (A, B) and day-10 (C, D) of growth. Square symbols represent non-acclimated strains and triangle symbols represent acclimated strains. Analysis was determined from three replicate spectra for each treatment (shown in Fig. S3). (E, F) Changes in carbohydrate/amide I (A) and lipid/amide I (B) ratio at day-10 of growth of non-acclimated and acclimated strains in wastewater as determined by FTIR spectroscopy. All data are means ±SE of

3 replicates. * (P < 0.05) denotes significant difference between acclimated and non-acclimated strains in wastewater.

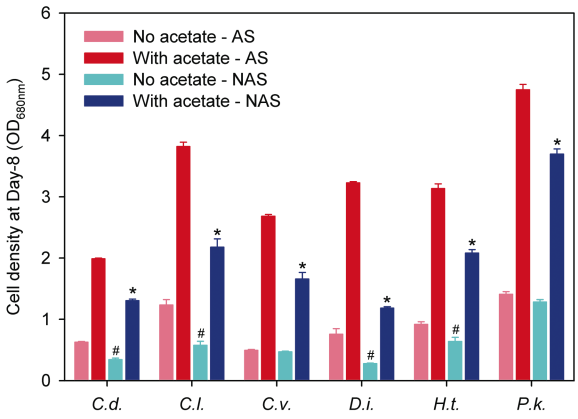

Fig. 4 Utilisation of acetate by acclimated and non-acclimated strains. Cell densities of acclimated strains (AS) and non-acclimated strains (NAS) of *C. debaryana* (*C.d.*), *C. luteoviridis* (*C.l.*), *C. vulgaris* (*C.v.*), *D. intermedius* (*D.i.*), *H. tetrachotoma* (*H.t.*) and *P. kessleri* (*P.k.*) grown in artificial medium either with or without acetate determined at day-8. All data are means ±SE of 3 replicates. * (P < 0.05) denotes significant difference between AS and NAS in medium with acetate. # (P < 0.05) denotes significant difference between AS and NAS in medium without acetate.

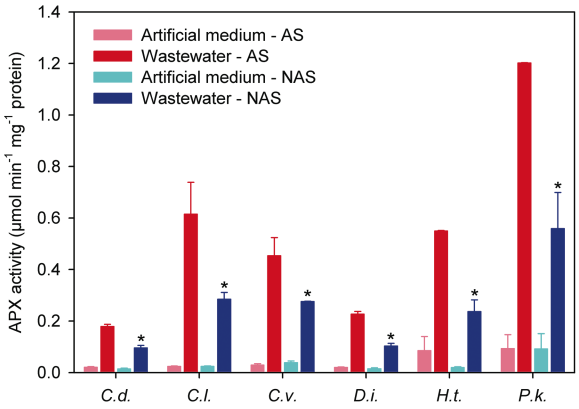

Fig. 5 Ascorbate peroxidase (APX) activity of acclimated and non-acclimated strains grown in wastewater. Acclimated strains (AS) and non-acclimated strains (NAS) of *C. debaryana* (*C.d.*), *C. luteoviridis* (*C.l.*), *C. vulgaris* (*C.v.*), *D. intermedius* (*D.i.*), *H. tetrachotoma* (*H.t.*) and *P. kessleri* (*P.k.*) grown in artificial medium were either transferred into wastewater or fresh artificial medium and APX activity was determined after 24 h exposure. All data are means ±SE of 3 replicates. * (P < 0.05) denotes significant difference between AS and NAS in wastewater.

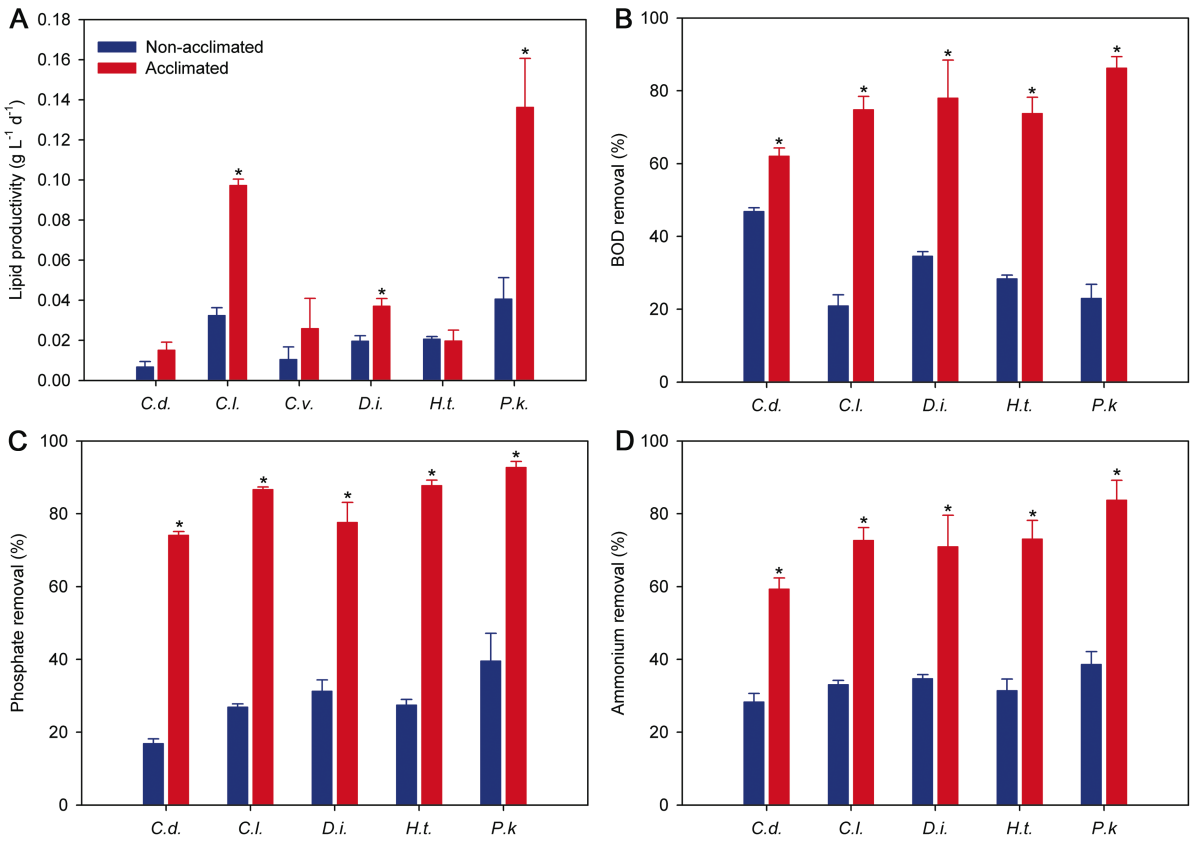

Fig. 6 Oil productivity and pollutant remediation of acclimated strains in wastewater. (A -D) Total lipid productivity determined during exponential growth phase (A), and reduction biochemical oxygen demand (BOD) (B), PO_4^{3-}-P (C), and NH_4^+-N (D) determined at day-10 of growth of non-acclimated and acclimated *C. debaryana* (*C.d.*), *C. luteoviridis* (*C.l.*), *C. vulgaris* (*C.v.*), *D. intermedius* (*D.i.*), *H. tetrachotoma* (*H.t.*) and *P. kessleri* (*P.k.*) strains in wastewater. All data are means ±SE of 3 replicates. * ($P < 0.05$) denotes significant difference between acclimated and non-acclimated strains in wastewater.